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13. ABSTRACT ***(Maximum 200 Words)***

Evidence suggests that sensitivity to chemotherapy is largely due to a functional apoptotic pathway. Thus, a better understanding of the signal transduction pathways that lead to rescue from apoptosis might lead to improved modalities of treatment for unresponsive cancer types. **The focus of our studies is to elucidate the role of p90^{rsk} in antagonizing apoptosis in breast cancer cells.** P90^{rsk} is a serine-threonine protein kinase in the Ras-Raf-ERK (extracellular signal-regulated kinase, also known as mitogen-activated protein kinase or MAP kinase) cascade that lies immediately downstream of ERK1/2. Although the Ras pathway and ERKs have been the focus of much research in the cancer field, less is known about the role of p90^{rsk}. We hypothesize that p90^{rsk} may be particularly relevant to breast cancer cell survival because evidence suggests it can not only directly phosphorylate and activate the estrogen receptor but also has the potential to antagonize apoptosis by phosphorylating and inactivating Bad, a proapoptotic Bcl family member. In these studies, we tested the hypothesis that p90^{rsk} may also have the ability to phosphorylate and inactivate the forkhead family of transcription factors, such as FKHRL1. Our preliminary results, presented here, suggest this may indeed be the case.

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INTRODUCTION

This annual report covers the period September 1st, 2001 through August 31st, 2002. The tasks outlined in the Statement of Work that are applicable to this funding period are Tasks 6 through 10, as below. I have included brief summaries of the work accomplished with data, where applicable.

Task 6: Determine the contribution of the PI3-kinase pathway v.s. the Ras-Raf-ERK pathway on p90^{rsk1} activation.

Below, in Figure 1, I demonstrate the activities of cell lines generated by transduction of the p90^{rsk1} alleles, wild-type (WT), constitutively active (CA), and kinase-dead (KD).

In Figure 2 (next page), I show that the inhibition of PI3-kinase in MCF-7 cells is significantly inhibited by wortmannin, an inhibitor of PI3-kinase.

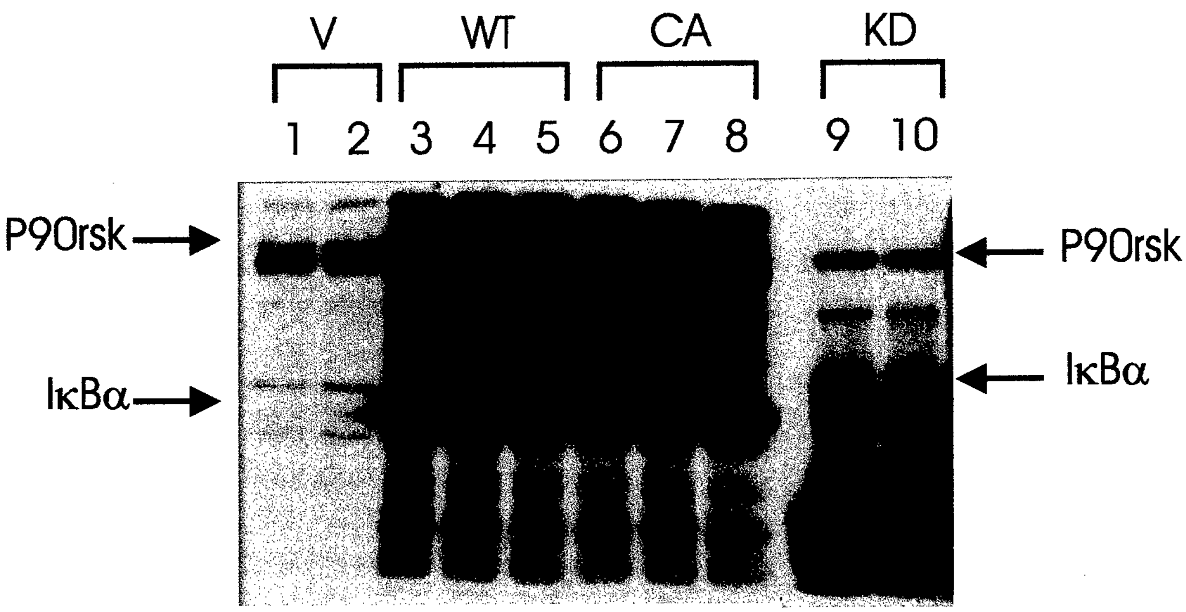


Figure 1: Immunoprecipitation-Linked Protein Kinase Assays of Cell Lines Expressing Alleles of p90rsk. Cells expressing vector (V), wild-type (WT), constitutively active (CA) and kinase-dead (KD) alleles of p90^{rsk} were immunoprecipitated with anti-HA antibody. The extracts analyzed were from: lane 1, HMV-4; 2, MCV-5; 3, HMWT-12; 4, MCWT-13; 5, MCWT-14; 6, HMCA-15; 7, HMCA-16; 8, MCCA-18; 9, HMKD-7; 10, MCKD-9. Lanes 1-8 are films of gels exposed for 1 hour. Lanes 9 & 10 were taken from a separate gel which was exposed for 18 hrs.

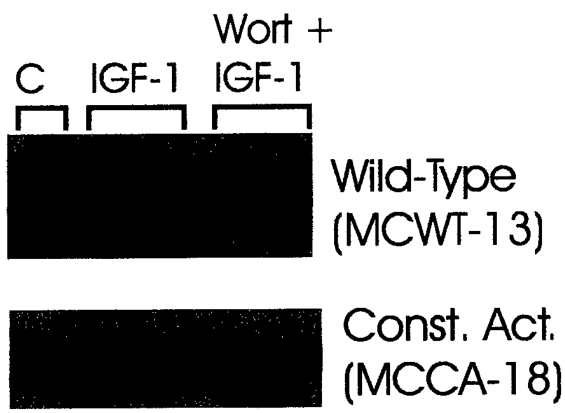
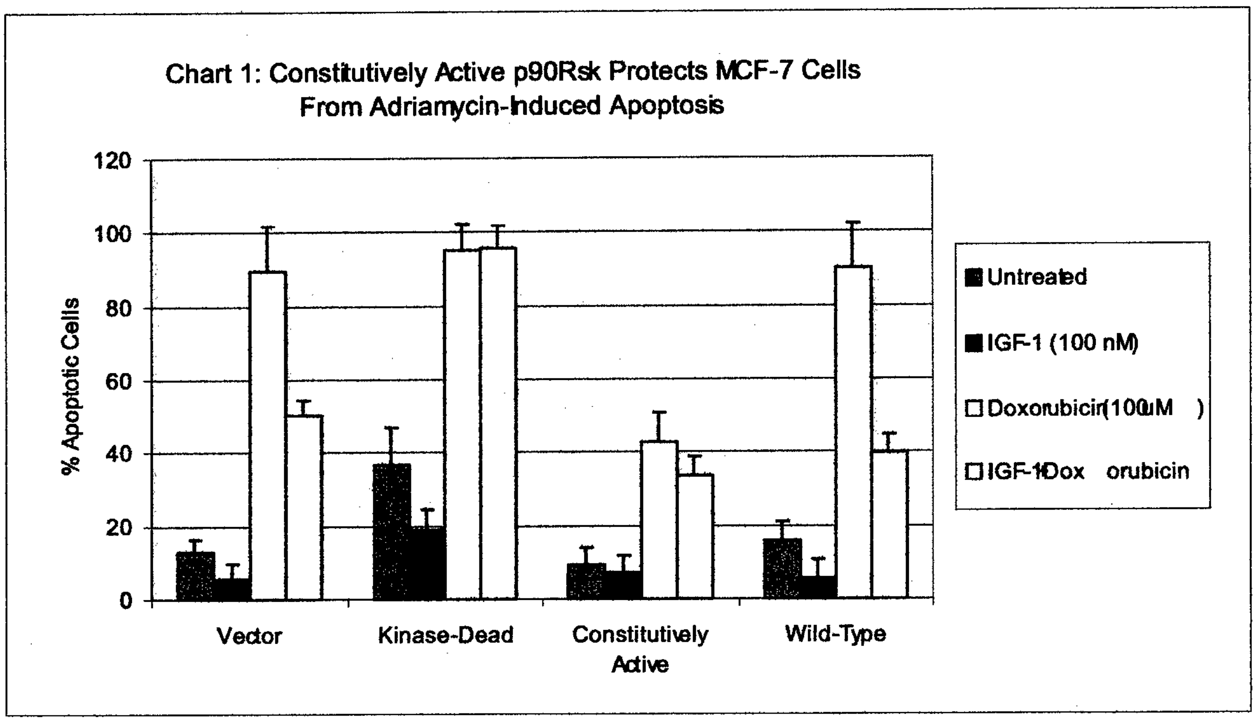


Figure 2: Immunoprecipitation-linked protein kinase activity of MCF-7 cell carrying WT and CA alleles of p90rsk treated with IGF-1 or IGF-1 and wortmannin. Control cells were starved overnight but not treated with either IGF-1 or wortmannin. Cells were either treated with 10 ng/ml of IGF-1 or IGF-1 and 200 nM wortmannin.

Task 7 & 8: Generate a dose-response curve for adriamycin in breast cancer cells and determination of apoptotic index.
Based on assays as represented below, we determined an optimal working concentration for doxorubicin (adriamycin). The expression of constitutively active or wild-type p90rsk significantly inhibited apoptosis.

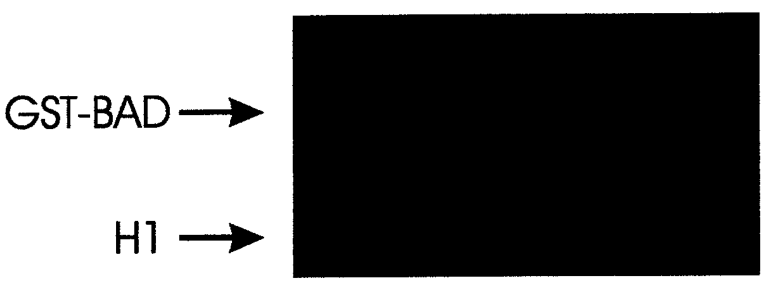


Task 9: Determine whether p90^{rsk1} phosphorylates ER and BAD.

In Figure 3, below, an *In vitro* immunoprecipitation-linked phosphorylation assay was performed using anti-p90rsk using GST-BAD and H1 as substrates. IGF-1(100ng/ml) was used to stimulate p90rsk activity (lanes 3,4,6). Wortmannin (10 uM) was used to inhibit PI3-kinase (lanes 2,4). Cells carrying the kinase-dead construct was assayed for BAD phosphorylating activity in lanes 5 and 6.

Figure 3: *In vitro* phosphorylation of GST-BAD by immunoprecipitated endogenous p90^{rsk}.

IGF-1	–	–	+	+	–	+
Wortmannin	–	+	–	+	–	–
KD-p90rsk	–	–	–	–	+	+
	1	2	3	4	5	6



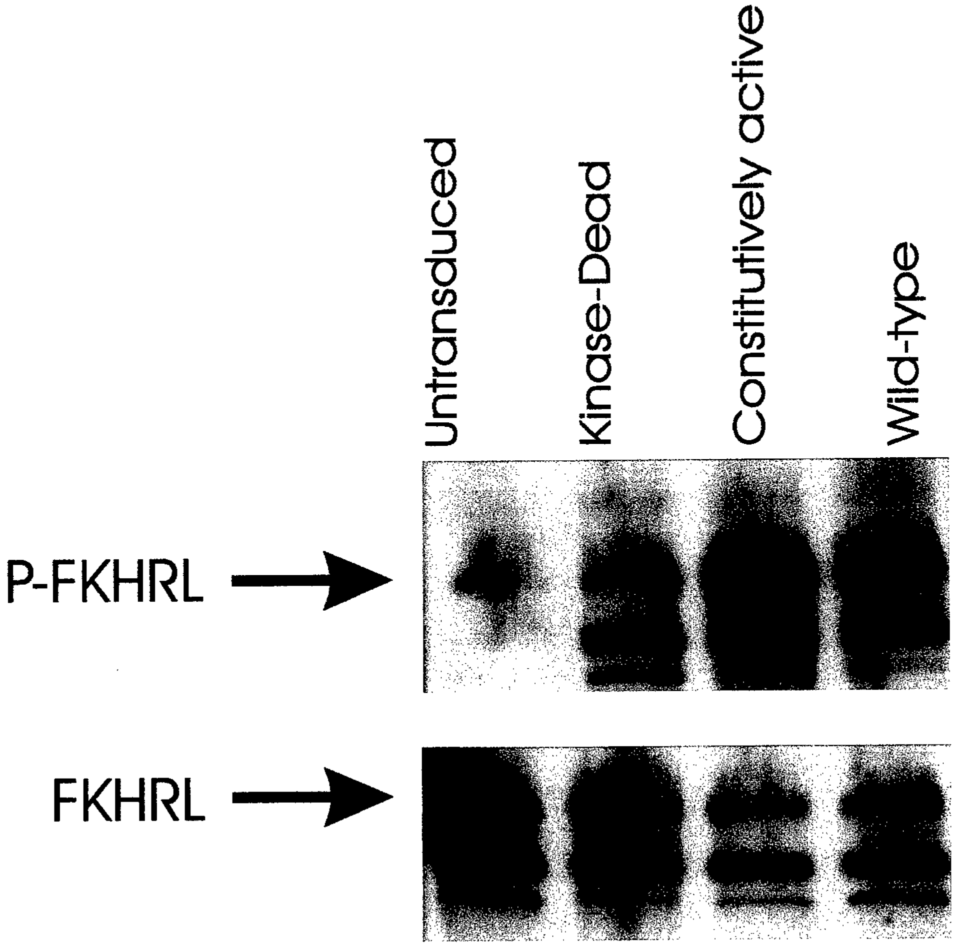


Figure 4: Forkhead phosphorylation can be regulated by p90rsk. MCF-7 cells were treated with 100 nM IGF-1 for 30 min and harvested for westerns. Top panel, anit-phospho-FKHRL1 antibody treatment of vector, kinase-dead, constitutively active or wild-type p90rsk -transduced cells. Bottom panel, the same extracts probed for total FKHRL.

Task 9: Determine whether p90^{rsk1} phosphorylates ER and BAD.

At present, my laboratory is still optimizing our methodology for site-specific phosphorylation of ER. In the meantime, I have included data on another recently identified potential substrate of p90rsk, Forkhead. Forkhead (FKHRL1 was investigaged in this case) is a transcription factor that was originally identified in *C. elegans* as a downtream target of insulin/IGF-1 signalling [Lee, et al., 2001]. Mutations in the insulin/IGF-1 like pathway or the Forkhead transcription factor that sequestered it to the cytoplasm following phosphorylation render the worm long-lived and resistant to stress.

Task 10: Determine whether cotransfection of p90^{rsk1} and BAD rescues apoptosis mediated by BAD.

Work on this specific aim is ongoing. My laboratory is establishing a collaboration with a lab that has established some knockout embryonic stem cells that lack Akt. This should enhance our ability to study BAD phosphorylation in the cell and help us analyze the data obtained from constitutively active transduction of p90rsk.

Reportable Outcomes

All evidence points to an anti-apoptotic role for p90rsk.

Conclusions

- Inhibition of PI-3 kinase has variable effects on $P90^{rsk}$. In some cases it appears to inhibit activation by IGF-1 but in other cases the inhibition is not complete.
- Enzymatically active or activatable forms of p90rsk can protect cells from adriamycin-induced apoptosis.
- GST-BAD is phosphorylated by p90rsk in an in vitro kinase assay using endogenous immunoprecipitated p90rsk.
- A kinase-dead allele of p90rsk inhibits the phosphorylation of GST-BAD by p90rsk.
- The forkhead transcription factor, FKHRL1, is phosphorylated by transduction of a constitutively active allel of p90rsk.

References

Lee, R. Y., J. Hench, and G. Ruvkun 2001. Regulation of C. elegans DAF-16 and its human ortholog FKHRL1 by the daf-2 insulin-like signaling pathway Curr Biol. **11:**1950-7.

Appendices

- None included.